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Metabolic adaptations and changes of the mammary immune response during beta-hydroxybutyrate administration

PhD Thesis submitted by

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1. Abstract

Elevation of ketone bodies occurs frequently after parturition during negative energy balance in high yielding dairy cows. Previous studies illustrated that hyperketonemia interferes with metabolism and it is assumed that it impairs the immune response. However, a causative effect of ketone bodies could not be shown in vivo before, because spontaneous hyperketonemia comes usually along with high NEFA and low glucose concentrations. The objective was to study effects of beta-hydroxybutyrate (BHBA) infusion and an additional intramammary lipopolysaccharide (LPS) challenge on metabolism and immune response in dairy cows. Thirteen dairy cows received intravenously either a BHBA infusion (group BHBA, n=5) to induce hyperketonemia (1.7 mmol/L), or an infusion with a 0.9 % saline solution (Control, n=8) for 56 h. Infusions started at 0900 on day 1 and continue up to 1700 two days later. Two udder quarters were challenged with 200 µg Escherichia coli-LPS 48 h after the start of infusion. Blood samples were taken one week and 2 h before the start of infusions as reference samples and hourly during the infusion. Liver and mammary gland biopsies were taken one week before the start of the infusion, 48 h after the start of the infusion, and mammary tissues was additionally taken 8 h after LPS challenge (56 h after the start of infusions). Rectal temperature (RT) and somatic cell count (SCC) was measured before and 48 h after the start of infusions and hourly during LPS challenge. Blood samples were analyzed for plasma glucose, BHBA, NEFA, triglyceride, urea, insulin, glucagon, and cortisol concentration. The mRNA abundance of factors related to potential adaptations of metabolism and immune system was measured in liver and mammary tissue biopsies. Differences between blood constituents, RT, SCC, and mRNA abundance before and 48 h after the start of infusions, and differences between mRNA abundance before and after LPS challenges were tested for significance by GLM of SAS procedure with treatment as fixed effect.

Area under the curve was calculated for blood variables during 48 h BHBA infusion and during the LPS challenge, and additionally for RT and SCC during the LPS challenge. Most surprisingly, both plasma glucose and glucagon concentration decreased during the 48 h of BHBA infusion ($P<0.05$). During the 48 h of BHBA infusion, serum amyloid A mRNA abundance in mammary gland was increased ($P<0.01$), and haptoglobin (Hp) mRNA abundance tended to increase in cows treated with BHBA compared to control group ($P= 0.07$). RT, SCC, and candidate genes related to immune response in the liver were not affected by BHBA infusion. However, during LPS challenge the expected increase of both plasma glucose and glucagon concentration was much less pronounced in the animals treated with BHBA ($P<0.05$) and also SCC increased much less pronounced in the animals infused with BHBA ($P<0.05$) than in the controls. An increased BHBA infusion rate to maintain plasma BHBA constant could not fully compensate for the decreased plasma BHBA during the LPS challenge which indicates that BHBA is used as an energy source during the immune response. In addition, BHBA infused animals showed a more pronounced increase of mRNA abundance of IL-8, IL-10, and citrate synthase in the mammary tissue of LPS challenged quarters ($P<0.05$) than control animals. Results demonstrate that infusion of BHBA affects metabolism through decreased plasma glucose concentration which is likely related to a decreased release of glucagon during hyperketonemia and during additional inflammation. It also affects the systemic and mammary immune response which may reflect the increased susceptibility for mastitis during spontaneous hyperketonemia. The obviously reduced gluconeogenesis in response to BHBA infusion may be a mechanism to stimulated the use of BHBA as an energy source instead of glucose, and/or to save oxaloacetate for the citric acid cycle instead of gluconeogenesis and as a consequence to reduce ketogenesis.

2. Introduction

Since decades dairy cows have been selected for high milk production. High yielding dairy cows need a lot of energy and nutrients for maintenance and milk synthesis in particular during peak lactation. Despite of increased feed intake in the early lactation (Agenäs et al., 2003) after the periparturient nadir, the adaptation cannot cover the requirements during this period. A negative energy balance (NEB) at the onset of lactation is the consequence (van Dorland et al., 2009; Gross et al., 2011). Typical metabolic changes during this period of negative energy balance are low plasma concentrations of glucose, and high concentrations of plasma non-esterified fatty acid (NEFA) and subsequently elevated ketone bodies (van Dorland et al., 2009; Gross et al., 2011). Concurrently with the increase in milk production, the incidence of infectious diseases increased (Simianer et al., 1991; Syvajarvi at al., 1986; Uribe at al., 1995). Mainly during the first weeks of lactation cows experience an immunosuppression and high susceptibility of infectious diseases (Smith et al., 1985; Hogan et al., 1989; Goff, 2006) which is supposed to be due to the metabolic adaptations to negative energy balance (NEB) (Suriyasathaporn et al., 2000; van Dorland., 2009).

2.1 Hyperketonemia

The concentration of plasma BHBA, which is the major circulating ketone body in ruminants, is increased as a compensatory response to handle the excessive NEFA release during the NEB (van Dorland et al., 2009; Gross et al., 2011). Increased plasma BHBA concentrations above 1200 µmol/L are considered as a metabolic disorder (Ospina et al., 2010) defined as subclinical ketosis (Duffield et al., 2009). An elevation of plasma BHBA concentration beyond the threshold of 1,200 µmol/L (Ospina et al., 2010) is generally accepted to represent the diagnosis of a subclinical ketosis (Duffield et al., 2009).

2.2 Role of ketone bodies in the tissues

Beta-hydroxybutyrate can serve as an energy source in many tissues, especially during NEB, and it used for citrate synthesis in the mammary gland (Bionaz and Loor, 2008). Although ketone bodies can be used as an alternative fuel for some tissues such as brain and heart (Laffel, 1999; Veech, 2004), kidney (Weidemann and Krebs, 1969), skeletal muscles (Ruderman and Goodman, 1973) and lactating mammary gland (Shaw, 1943), the utilization of ketone bodies is limited. Unused ketone bodies accumulate and their concentration increases in blood (Duffield et al., 2009). This elevation of ketone bodies in blood causes reduced feed intake and increases the risk of clinical ketosis (CK), displaced abomasums (DA), metritis and subsequent decrease of milk production (Duffield et al, 2009). The mostly used method to detect of subclinical ketosis is the measurement of BHBA in serum or plasma (Duffield, 2000; Herdt, 2000). In ruminants, BHBA is the major ketone body found in circulation (Bergman, 1971). Ospina et al. (2010) suggested a threshold value of 1200 µmol/L to distinguish between normal cows and subclinically ketotic cows.

2.3 Effects of hyperketonemia on feed intake

Beta-hydroxybutyrate can play a role in the regulation of feed intake (Langhans, 1983). Subcutaneous injection of BHBA (10 mmol/kg body weight) in rats (Langhans et al., 1983; Moor et al., 1976), intraperitoneally BHBA infusion (15 mmol/kg$^{0.75}$) in pigmy goats (Rossi et al., 2000), and intracerebroventricular BHBA infusion (18mM/d) in dairy cows (Kuhla, 2011) decreased feed intake. In an in vitro experiment, elevation of BHBA: glucose ratio to 1:1 in early lactation compare with non-ketotic cows with a 1:4 ratio, can decrease feed intake via stimulation of dephosphorylation of AMPK after parturition (Laeger et al, 2012).

2.4 Effects of hyperketonemia on metabolism

High elevation of plasma BHBA concentration beyond the physiological range impairs metabolism in animals (Müller et al., 1984; Schlumbohm and Harmeyer, 2004). Intravenous BHBA infusion lead to a decreased plasma concentration of glucose in dogs (Madison et al., 1964) pigs (Müller et al., 1984), and ewes (Schlumbohm and Harmeyer, 2003, 2004). Information about the reasons for decreased plasma glucose concentration as a response to elevated BHBA concentration is limited. Hypothetically, insulin plays a role in this effect. Infusion of BHBA increases insulin secretion in pigs (Müller 1984), and Madison et al (1964) suggested that pancreatic beta cells respond to the high plasma BHBA concentration with increased insulin secretion in dogs. It appears that insulin suppresses the glucose production through an inhibitory effect on the regulatory enzymes of gluconeogenesis (Hayirli, 2006; Brockman and Larveld, 1986). This release of endogenous insulin can lead to a decline in plasma glucose concentration, and reduce the hepatic glucose production. In fact the ketone bodies represent only a modest stimulus on insulin secretion in ruminant (Jordan and Philips, 1978; Heitman and Fernandez, 1986). Insulin did obviously not play a significant role in mediating the lowering effect of BHBA on plasma glucose concentration in sheep (Schlumbohm and Harmeyer 2003). Decreased glucose concentration and hepatic glucose output due to the infusion of BHBA can reduce the availability of glucose for the peripheral tissues because exhausted glucose stores must be replaced by a catabolism of proteins during emergency conditions for the vital tissues such as central nervous system (Madison et al., 1964). The molarity of ketone bodies in plasma are the same or higher than that of glucose. Thus, enhanced plasma BHBA concentration can increase the efficiency of BHBA as a competitor to glucose used by peripheral tissues and has been shown to inhibit peripheral glucose utilization (Madison et al., 1964). Mebane et al (1962)

demonstrated that ketone body infusion leads to a 30% reduction in glucose utilization by peripheral tissues. Previous studies also reported that the oxidation of glucose was inhibited by ketone body infusion (Williamson and Krebs, 1961). In contrast, as above mentioned Schlumbohm and Harmeyer (2004) suggested that BHBA infusion decreased the endogenous glucose production but did not affect glucose utilization in pregnant sheep. Furthermore they suggested that the elevated concentration of ketone bodies in blood initially decline glucose production via a depressed stimulation of hepatic gluconeogenesis. Müller et al (1984) suggested that ketone bodies probably have a direct inhibitory action on gluconeogenesis. Soling and Kleineke (1976) suggested that ketone bodies have a glucose sparing effect in tissues through mitochondrial formation of citrate, an allosteric inhibitor, phosphofructo-1,6-kinase, acetyl- CoA inhibition of pyruvate dehydrogenase and activation of pyruvate carboxylase.

2.5 Effect of hyperketonemia on mastitis incidence and immune responses

The elevation of plasma ketone body concentration increases the risk of clinical ketosis, displaced abomasum, metritis and subsequent decrease of milk production (Duffield et al., 2009). Bovine mastitis is a complex and costly disease in high yielding dairy farming, which contributes to decreased milk production from 110 to 552 kg per lactation period and often cows do not return to their pre-mastitis production (Rajala-Schultz et al., 1999). Milk somatic cells are an indicator of the inflammatory response (Schukken et al., 2003), and elevated SCC is worldwide accepted as a sign of mastitis in dairy cows (Daley et al., 1991; Newbould and Neave, 1965). Macrophages, lymphocytes, and few neutrophils and epithelial cells are the components of milk somatic cells in healthy mammary glands, while neutrophils present the main cell population (>95%) in infected mammary glands (Philpot and Nickerson, 1991; Kehrli et al., 1994). Increase neutrophil and subsequent SCC is a mechanism defense against the mammary gland infection

(Sandholm et al., 1995). The occurrence of mastitis is determined by the number of pathogenic organisms, the animal, and environmental factors (Burvenich et al., 2007). Among the animal factors high plasma BHBA concentration has negative influence for susceptibility of mastitis and course of disease (Heyneman et al., 1990; Van Werven et al., 1997; Oltenacu and Ekesbo 1994). In addition, the elevation of plasma BHBA concentration decreased the chemotaxis and microbial killing in human in vitro (McMurray et al., 1990). Elevated plasma BHBA concentration had a positive correlation with the severity of *E. coli* mastitis in an in vitro study (Kremer et al., 1993), and the risk of mastitis was much higher in subclinically ketotic dairy cows (Oltenacu and Ekesbo, 1994). Duffield et al. (1998) reported an association between hyperketonemia and increased SCC after parturition in dairy cows. An increase of SCC is accepted as an indicator of inflammatory responses in the mammary gland (Schukken et al., 2003; Pfaffl et al., 2003; Wellnitz et al., 2011). Earlier in vitro studies illustrated that elevation of BHBA concentration decreased the chemotactic response of neutrophils in bovine milk leukocytes *in vitro* (Cerone et al., 2007; Klucinski et al., 1988).

Cytokine production was reduced after bacterial infection in ketotic dairy cows (Kandefer-Szerszen et al., 1992; Filar et al., 1992). However, elevated BHBA occurs mostly concomitantly with other metabolic and endocrine changes (Kessel et al., 2008; Gross et al., 2011), and the immunosuppressive effect cannot be exclusively ascribed to the ketone bodies. Results of exclusive BHBA effects on the immune system are available from in vitro studies. In the presence of BHBA a decreased phagocytotic activity of milk neutrophils (Klucinski et al., 1988) and a reduced chemotactic capacity of bovine blood leukocytes (Suriyasathaporn et al., 1999) were demonstrated. There is evidence that ketone bodies, specifically acetoacetate, increases IL-8, and IL-6 concentration in human U937 and THP-1 monocyte cell lines and human umbilical

vein endothelial cells, while the presence of BHBA does not affect these cytokine concentrations (Rains et al., 2011; Jain et al., 2007). IL-8 is a chemokine that is produced by lymphocytes (Gregory et al., 1988), neutrophils (Strieter et al., 1990), and epithelial cells (Skansen-Saphir et al., 1993). IL-8 is involved into the recruitment of neutrophils and activates them (Harda et al., 1994) during mastitis (Barber and Yang, 1998) and mammary epithelial cells secrete IL-8 in response to LPS (Wellnitz and Kerr, 2004).

Information about the effects of hyperketonemia isolated from other metabolic changes on the immune response and SCC in hyperketotic cows is rare, and the effects of long term hyperketonemia have not been investigated in dairy cows.

The use of intramammary LPS challenge to simulate intramammary infection and to induce mastitis in dairy cows was established previously (Bruckmaier et al., 1993) to investigate the effect of mastitis on metabolism, immune responses, and performance in dairy cows. There is evidence that LPS challenge affects metabolism and mRNA abundance of inflammatory and other factors (Waldron et al., 2006; Bruckmaier et al., 1993; Vernay et al., 2012). Schmitz et al. (2004) illustrated that intramammary LPS stimulation has also an influence on systemic immune response.

2. 6 Objective

Our objective was to induce an elevated plasma BHBA concentration over 56 hours trough BHBA infusion, without the metabolic situation as observed during the early lactation, and additionally stimulate the immune system by intramammary LPS challenge in mid-lactation dairy cows to investigate:

- The effects of BHBA elevation over 48 h on feed intake, performance, and metabolism.
- More specifically, this study was carried out to confirm that also in the dairy cows, like in sheep and pig, elevated plasma BHBA concentration affects plasma glucose concentration, and to investigate the underlying mechanisms.
- The effects of long term elevation of plasma BHBA (48 h) on mRNA abundance related to the metabolism and immune responses in the liver and mammary gland.
- Systemic metabolic effects and changes of the mRNA abundance of genes related to metabolism in mammary tissue during the LPS challenge.
- The effects of elevated BHBA on SCC and mammary immune response to LPS challenge during for additional 8 h.

3. Results

Long-term elevation of beta-hydroxybutyrate in dairy cows through infusion: effects on feed intake, milk production, and metabolism

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Hyperketonemia during LPS induced mastitis affects systemic and local intramammary metabolism in dairy cows

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Induced hyperketonemia affects the mammary immune response during lipopolysaccharide challenge in dairy cows

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4. Discussion

This study is to the best of our knowledge the first that induced an elevated plasma BHBA concentration for long time (56 h) in dairy cows by infusion of BHBA. Plasma BHBA concentration during 48 h reached was 1.7 ± 0.1 mmol/L, which was clearly above the mostly considered concentration of 1.2 to 1.4 mmol/L referring to subclinical ketosis (Duffield et al., 2009; Ospina et al., 2010). The goal to maintain plasma BHBA at 1.7 mmol/L through an increased BHBA infusion rate after LPS challenge (8 h) could not be achieved because the decline of plasma BHBA was faster than the adjustment of infusion rate was possible.

4.1 Effect of hyperketonemia on feed intake, milk yield, and energy balance

The expected reduction of DMI in HyperB cows as shown in cows with spontaneously elevated ketone body concentrations (Bareille et al., 2003; Gonzàlez et al., 2008) was not observed in our study. In our study, BHBA infusion did not affect feed intake throughout the experiment which may relate to low ratio of BHBA: glucose. It can be speculated that low feed intake during NEB is related to low plasma glucose concentration that causes to decrease appetite in this period, while the low plasma glucose concentration accrued follow the BHBA, in a positive energy balance in the present study.

The lacking effect on milk synthesis was likely due to the related low needs of glucose for mammary lactose synthesis and other metabolic processes at this lactational stage compared to early lactation. Thus the reduced plasma glucose levels during BHBA infusion were likely not limiting for milk secretion.

4.2 Effect of hyperketonemia on metabolism

During the 48 h BHBA infusion plasma glucose concentration decreased dramatically. The finding of the present study is consistent with other reports in pregnant sheep (Schlumbohm and Harmyer 2003, 2004), dogs (Madison et al., 1964; Felts et al., 1964), and pigs (Müller et al, 1984). We suspect that the decrease in glucose concentration in our study is related to elevated BHBA concentration as BHBA is used as an alternative fuel and energy source for the tissues, or a decline of glucagon secretion through the effect on glycogenolysis or gluconeogenesis during the experiment. There is evidence that glucagon secretion is inhibited by the neighboring β- cell through insulin (Weir et al, 1976) and gamma amino butyric acid (GABA) (Adeghate et al., 2000; Wendt et al., 2004). Gamma amino butyric acid is an inhibitory neurotransmitter in the brain that increased in present of high concentration of BHBA in an in vitro study in the epileptic brain (Suzuki et al., 2009), and has an inhibitory effect on glucagon secretion. A transient increase and a subsequent decrease of plasma glucose concentration after LPS challenge in both groups in this study was consistent with previous studies that illustrated a transient hyperglycemia after *E. coli* endotoxin induced mastitis (Bruckmaier et al., 1993), increased and subsequent decreased plasma glucose concentration after LPS challenge (Werling et al., 1996). Regarding to increase plasma glucose concentration after the LPS challenge, during a similar immune stimulation via LPS challenge previously, development of an insulin resistance was reported, and in this case glucose infusion rate had to be reduced to avoid an increase of plasma glucose concentration, thus additional glucose was available through either glycogenolysis or gluconeogenesis (Vernay et al., 2012). Based on the present data it seems that the initial increase plasma glucose concentration after LPS challenge is not related to the glycogenolysis, because LPS challenge increased plasma glucagon concentration 150 min after the LPS administration.

The difference between plasma glucose concentrations in the two experimental groups after the LPS challenge is related to the BHBA infusion that decreased glucose production besides the decreased gluconeogenesis during LPS. The low plasma glucose concentration in HyperB rather than in the control group may be related to the reduced increase of plasma glucagon concentration in HyperB compared to the control group which resulted in low glucose production from gluconeogenesis or glycogen storage.

It seems that unchanged plasma NEFA concentration in response to the BHBA infusion (48 h) and LPS challenge in the present study is related to utilize BHBA instead of glucose by peripheral tissues (Madison et al., 1964), which can diminish fatty acid mobilization from adipose tissues. On the other hand, after a decline in glucose concentration and subsequently NEB, increase of NEFA occurred in early lactation (van Dorland et al., 2009), but in this study despite of decreased glucose, cows were not in the NEB situation.

Intramammary LPS challenge decreased plasma BHBA in both treatment groups. Because the decline of BHBA after LPS administration was quite rapid and pronounced a contribution of changed ruminal absorption may be excluded. Based on the fast and huge changes of plasma BHBA in BHBA infused animals in the present study decreased hepatic ketogenesis capacity (Memon et al., 1992) seems very unlikely. Recent findings showed that LPS challenge increased milk BHBA concentration in LPS treated quarters during an induced hyperketonemia (Lehmann et al., 2013). Thus a certain quantity of BHBA is lost with the secreted milk in LPS stimulated quarters. However, this portion does not seem to be quantitatively of great importance because milk secretion is reduced in the quarters treated with LPS. It can be assumed that in the present study the rapid decline of plasma BHBA concentration following the LPS challenge, despite the

marked increase of BHBA infusion rate, is related to the use of BHBA as an alternative energy source for immune system activity.

In the current study increased plasma glucagon concentration in both groups was observed at 150 min after the intramammary LPS administration. However, the increase of plasma glucagon concentration during LPS challenge was much less in cows that received BHBA compared to Control. The less pronounced increase of glucagon during LPS challenge in the HyperB group is likely related to a lower need of glucose for the immune response than in the control group, and thus less activation of gluconeogenesis.

The unchanged insulin concentration in response to the BHBA infusion (48 h) in the present study may be explained by the fact that ketone bodies represent only a modest stimulus on insulin secretion in ruminant (Jordan and Philips, 1978; Heitman and Fernandez, 1986). An increased plasma insulin concentration in response to the LPS challenge in the present study is in agreement with previous studies (Waldron et al., 2003; Waldron et al., 2006; Vernay et al., 2012). The effect of LPS challenge on glucoregulatory hormones is most likely related to the effects of pro inflammatory cytokines (Eizirik et al., 1995; Andersson et al., 2001) which are stimulating the pancreatic production and release of these hormones.

As previously observed in response to intramammary LPS administration (Lehtolainen et al., 2003; Waldron et al., 2006; Vernay et al., 2012), plasma cortisol concentration increased in both treatment groups. Pro-inflammatory cytokines and possibly also the handling of the animals during the experiments likely activated the hypothalamus-pituitary-gland axis and increased the synthesis of glucocorticoids (Beishuizen and Thijs, 2003). Elevation of plasma cortisol

concentration may be partially responsible for the observed induction of an insulin resistance (Andrews and Walker, 1999) to provide more glucose for the immune reaction.

4.3 Effects of elevated BHBA concentration on mRNA abundances of genes related to metabolism

Our results of mRNA abundance of key enzymes related to hepatic gluconeogenesis (pyruvate carboxylase, glucose-6-phosphatase, mitochondrial phosphoenolpyruvate carboxykinase) are in contrast with suggestions from previous studies that indicated an inhibition of gluconeogenesis by elevated plasma BHBA concentration (Schlumbohm and Harmyer, 2003; Müller 1984). Beta-hydroxybutyrate infusion did not change the key enzymes related to gluconeogenesis, glycolysis, pyruvate dehydrogenase complex (PDH complex), and citrate synthase mRNA expression. According to the mRNA abundance of some enzymes related to fatty acid oxidation, BHBA infusion did not affect fatty acid oxidation. These results are not in agreement with previous reports (Soling and Kleineke, 1976; Morio and Wolfe, 2005) that mentioned ketone bodies are an inhibitor for fatty acid oxidation through increased acetyl CoA production, inhibited CPT1 activity, and stimulated malonyl CoA synthesis.

As mentioned above, ketone bodies caused a reduced glucagon secretion, and this inhibition could have been regulated via cAMP. The mechanism of this inhibition is related to the generation of ATP/ADP by substrate such as ketone bodies, then decrease of cAMP and finally prevention of glucagon secretion (Gerich et al., 1976; Toyota et al., 1975; Mitrakou et al., 1991; Adeghate et al., 2000). In the present study, the AMP-activated α 1 (PRKAA1) expression tended to decrease in HyperB compared with control group ($P<0.1$). On the other hand, BHBA infusion decreased the PRKAA1 expression, subsequently glucagon excretion was decreased.

The effect of the intramammary LPS challenge on FASN, OXCT1, and BDH2 mRNA abundance in quarters stimulated with LPS in both groups, and decrease of CS mRNA abundance in the control group showed that LPS challenge negatively affects mammary gland metabolism and milk synthesis, which had been reported before (Waldron et al., 2003; Waldron et al., 2006). The increase of CS mRNA abundance in HyperB after LPS challenge is in agreement with Bionaz and Loor (2008) who suggested that the major product of BHBA metabolism in the bovine mammary gland is citrate which is increased in milk. It can be assumed that the up-regulation of mammary gland CS mRNA abundance in HyperB group confirmed that BHBA can be partly used to produce energy through the tricarboxylic acid cycle by mammary gland (Palmquist et al., 1969).

4.4 Beta-hydroxybutyrate infusion effects on immune response

4.4.1 Hyperketonemia effects on somatic cell in milk

Unchanged SCC in milk during 48 h of increased BHBA concentrations in blood in the present study was in contrast to a previous study (Duffield et al., 1998) that reported an association between hyperketonemia and increased SCC after parturition in dairy cows. However, the BHBA infusion represented an additional energy source during already positive energy balance in the present study as compared to the NEB that was reported in the mentioned study. In addition, in the present study the induced hyperketonemia by BHBA infusion was completely different to spontaneous hyperketonemia during NEB that is accompanied by low plasma glucose concentration and high plasma NEFA concentration after parturition (Kessel et al., 2008; van Dorland et al., 2009; Gross et al., 2011).

Consistently with previous studies, LPS challenge caused the increase of body temperature (Schmitz et al., 2004; Vernay et al., 2012) and milk SCC (Wellnitz et al., 2011; Vernay et al., 2012). The induced increase of RT confirmed that LPS challenge caused not only a local activation of the immune response but had also a systemic effect (Dinarello, 1991). Interestingly, the increase of milk somatic cells was less pronounced in LPS quarters in HyperB than the control group. This is supported by earlier in vitro studies that illustrated that elevation of BHBA concentration decreased neutrophil chemotactic response in bovine milk leukocytes *in vitro* (Cerone et al., 2007; Klucinski et al., 1988). Therefore, it can be assumed that the diminished increase of SCC in the present study in the HyperB group compared to the control group is related to the negative effects of plasma BHBA concentration on neutrophil recruitment.

4.4.2 Effect of hyperketonemia on gene abundances related to immune response

Serum amyloid A mRNA abundance in mammary tissue was increased by long term BHBA (48 h) infusion, and Hp tended to increase in HyperB compared to NaCl in the present study. Beta-hydroxybutyrate infusion (48 h) increased hepatic SAA and Hp mRNA abundance in both treatment groups. The different effects of BHBA infusion on SAA and Hp mRNA abundance in the mammary tissue is most likely related to a higher sensitivity of SAA than Hp (Gruys et al., 1993; Alsemgeest et al., 1994; Werling et al., 1996). It can be speculated that the different effect of high plasma BHBA concentration (48 h) on acute phase protein mRNA abundance in the liver and in the udder was related to the role of the liver in the systemic immune response whereas the udder tissue acts mainly locally within the organ. This result demonstrates that increased BHBA concentrations in blood by infusions (48 h) affect the abundance of measured genes related to immune response in the udder but no in the liver.

Increased SAA and Hp mRNA abundance in mammary tissue in response to LPS challenge confirms previous studies that illustrated increased synthesis of SAA (Wellnitz and Kerr, 2004; Bruckmaier and Meyer, 2005; Vernay et al., 2012) and Hp (Hiss et al., 2004) in mammary tissue in response to LPS. Increased udder mRNA abundance of IL-1β, IL-6, iNOS, and TNFα, in quarters that were stimulated by intramammary LPS challenge in both groups is related to immune system activation and was documented in previous studies (Bruckmaier and Meyer, 2005; Wellnitz et al., 2011; Vernay et al., 2012; Hiss et al., 2004). Increased IL-6, IL-10, and Hp mRNA abundance in LPS quarters and control quarters in both treatment groups illustrated that intramammary LPS stimulation has also an influence on systemic immune response (Schmitz et al., 2004). Modified plasma BHBA concentration induced a more pronounced increase of IL-8 and IL-10 mRNA abundance compared to the control group, in LPS challenged quarters. It can be hypothesized that increased IL-8 mRNA abundance after LPS challenge is related to its role in the recruitment of immune cells from blood into milk. As BHBA infusion decreased neutrophil recruitment, the immune system was most likely forced to compensate the deficiency of BHBA effects on immune cell recruitment through the up-regulation of IL-8 mRNA abundance. It seems that up-regulation of IL-8, and IL-10 in HyperB group compared to the control group reflects negative effects of BHBA on the immune response.

5. Outlook

- Intravenous BHBA infusion successfully induced elevated plasma BHBA concentration.
- Feed intake is not affected by elevated BHBA concentration during a period positive energy balance.
- Decreased plasma glucose concentration during BHBA administration cannot be related to changes of insulin concentration or to mRNA abundance of genes related to hepatic gluconeogenesis.
- Reduced glucose concentration maybe related to decrease in plasma glucagon concentration which can decrease glucose production from gluconeogenesis or glycogen storage.
- Elevated plasma BHBA does not affect metabolism in the mammary gland and immune responses in the liver at a mRNA level.
- BHBA infusion affects systemic immune response and mammary immune response which may reflect the increased susceptibility for mastitis during spontaneous hyperketonemia.
- Induced hyperketonemia at LPS challenge: reduces the availability of glucose, likely via inhibiting glucagon release; causes reduced SCC increase in response to LPS; and affects cytokine secretion in the mammary tissue (based on mRNA).

The Results indicate that high ketone body concentration has impacts on glucose metabolism and mammary immune function.

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8. Overview of scientific communications

8.1 Peer review papers

Zarrin. M., O. Wellnitz, H.A. van Dorland, and R. M. Bruckmaier. Effect of manipulated metabolite changing on gene expressions of nuclear related factor-E2 in dairy cows (In process to submit to Journal of Animal Physiology and Animal Nutrition).

Zarrin. M., O. Wellnitz, and R. M. Bruckmaier. Short communication: Conjoint regulation of glucagon secretion via insulin and glucose plasma concentrations in dairy cows. (It is ready to submit to Journal of Domestic Animal Endocrinology).

Zarrin. M., O. Wellnitz, H.A. van Dorland, J. J. Gross, and R. M. Bruckmaier. 2014. Hyperketonemia during LPS induced mastitis affects systemic and local intramammary metabolism in dairy cows. J. Dairy Sci. doi.org/ 10.3168/jds.2013-7480.

Zarrin. M., O. Wellnitz, H.A. van Dorland, and R. M. Bruckmaier. 2014. Induced hyperketonemia affects the mammary immune response during lipopolysaccharide challenge in dairy cows. J. Dairy Sci. 97:330-339.

Zarrin, M., L. De Matteis, M. C. M. B. Vernay, O. Wellnitz, H.A. van Dorland, R.M. Bruckmaier. 2013. Long-term elevation of beta-hydroxybutyrate in dairy cows through infusion: effects on feed intake, milk production, and metabolism. J. Dairy sci. 96:2960-2972.

8.2 Contribution to scientific conferences

2011:

Zarrin, M., L. De Matteis, M. C. M. B. Vernay, O. Wellnitz, H.A. van Dorland, R.M. Bruckmaier. Induced hyperketonemia in dairy cows by long-term (48 h) BHB infusion and effects on milk yield and feed intake. 7th International Congress of Farm Animal Endocrinology. 2011. Bern, Switzerland.

2012:

Zarrin, M., H.A. van Dorland, O. Wellnitz, R. M. Bruckmaier. Effects of long-term beta-hydroxybutyrate (BHB) infusion on plasma glucose concentration in dairy cows. 5th Iranian animal science congress. 2012. Isfahan, I. R. Iran.

Zarrin, M., L. De Matteis, M. C. M. B. Vernay, O. Wellnitz, H.A. van Dorland, R.M. Bruckmaier. Effects of long term Hyperketonemia on metabolism and performance in lactating dairy cows.

ADSA – AMPA – ASAS – CSAS – WSASAS Joint Annual Meeting. 2012. Phoenix, Arizona, USA.

van Dorland, H.A., M. Zarrin, M.C.M.B. Vernay, O. Wellnitz, and R.M. Bruckmaier. Metabolic responses in lactating dairy cows to an infusion with DL-beta-hydroxybutyrate for 48 hours. Society of Nutrition Physiology. 2012. Gottingen, Germany.

2013:

Zarrin, M., O. Wellnitz, H.A. van Dorland, R.M. Bruckmaier. Long term hyperketonemia impairs metabolism and affects immune response in dairy cows were stimulated with LPS in dairy cows. 8th ECBHM Annual General Meeting. 2013. Bern, Switzerland.

Zarrin, M., O. Wellnitz, H.A. van Dorland, R.M. Bruckmaier. Conjoint regulation of glucagon secretion via insulin and glucose plasma concentrations in dairy cows. ADSA – ASAS Joint Annual Meeting. 2013. Indianapolis, USA.

Zarrin, M., H.A. van Dorland, O. Wellnitz, R.M. Bruckmaier. Effects of intravenous beta-hydroxybutyrate on the mRNA abundance of genes related to metabolism and immune response in hepatic and mammary tissue in dairy cows. ADSA–ASAS Joint Annual Meeting. 2013. Indianapolis, USA.

Zarrin, M., H.A. van Dorland, O. Wellnitz, R.M. Bruckmaier. Effects of an intramammary lipopolysaccharide (LPS) challenge on metabolism and mammary immune response in hyperketotic dairy cows. ADSA–ASAS Joint Annual Meeting. 2013. Indianapolis, USA.

Zarrin, M., H.A. van Dorland, R.M. Bruckmaier, O. Wellnitz. Effects of long term hyperketonemia combined with LPS challenge on immune response in dairy cows. 15th International Conference on Production Diseases in farm animals (ICPD), Book of abstracts (Göran Dallin, ed.), 24-28 June 2013, Uppsala, Sweden, pp 156.

Zarrin, M., J. J. Gross, O. Wellnitz, H.A. van Dorland, R.M. Bruckmaier. New findings on the effect of β-hydroxybutyrate on metabolism and immune response in dairy cows Are high performance "Bio-compatible"? Challenges for the animal Nutrition. ETH, Zurich. May 2013.

Zarrin, M., O. Wellnitz, H.A. van Dorland, R.M. Bruckmaier. Hyperketonemia affects metabolism and immune response in dairy cows. Swiss Association for Animal Production (SVT). 2013. Posieux, Switzerland.

Zarrin, M., H.A. van Dorland, O. Wellnitz, R.M. Bruckmaier. Effects of induced hyperketonemia on metabolism and mammary immune response in dairy cows. GCB symposium. 2013. Bern, Switzerland.

2014:

Zarrin, M., R.M. Bruckmaier, O. Wellnitz. Manipulated plasma insulin, glucose, and BHBA affect immune factors in somatic cells in milk with and without intramammary LPS challenge in dairy cows. ADSA-ASAS-CSAS Joint Annual Meeting, 2014. Kansas City, Missouri, USA.

Zarrin, M., O. Wellnitz, R. M. Bruckmaier. Nuclear related factor-E2 is down-regulated by hyperinsulinemic euglycemia in dairy cows ADSA-ASAS-CSAS Joint Annual Meeting, 2014. Kansas City, Missouri, USA.

Zarrin, M., O. Wellnitz, H.A. van Dorland, R.M. Bruckmaier. Blood glucose concentration is a major regulator of glucagon secretion in dairy cows. GCB symposium. 2014. Bern, Switzerland.

9. Appendix

Appendix I

Long-term elevation of beta-hydroxybutyrate in dairy cows through infusion: effects on feed intake, milk production, and metabolism

Journal of Dairy Science, Volume 96, No. 5, May 2013, Pages 2960-2972, doi:10.3168/jds.2012-6224

Zarrin, M., L. De Matteis, M. C. M. B. Vernay, O. Wellnitz, H.A. van Dorland, R.M. Bruckmaier.

Appendix II

Hyperketonemia during LPS induced mastitis affects systemic and local intramammary metabolism in dairy cows

Journal of Dairy Science, Volume 97, No. 6, June 2014, Pages 3531-3541, doi:10.3168/jds.2013-7480

Zarrin, M., O. Wellnitz, H.A. van Dorland, J.J. Gross, R.M. Bruckmaier.

Appendix III

Induced hyperketonemia affects the mammary immune response during lipopolysaccharide challenge in dairy cows

Journal of Dairy Science, Volume 97, No. 1, January 2014, pages 330-339, doi:10.3168/jds.2013-7222

Zarrin, M., O. Wellnitz, H.A. van Dorland, R.M. Bruckmaier.

Declaration of Originality

Last name, first name: Zarrin Mousa

Matriculation number: 11-104-445

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.

All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.

I am aware that in case of non-compliance, the Senate is entitled to withdraw the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 69, of 7 June 2011.

Place, date
Bern, 09.07.2014

Signature